

## Anaerobic microbial LCFA degradation in bioreactors

D.Z. Sousa\*, M.A. Pereira\*, J.I. Alves\*, H. Smidt\*\*, A.J.M. Stams\*\*, M.M. Alves\*

\* Institute for Biotechnology and Bioengineering, Center for Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

(E-mail: [dianasousa@deb.uminho.pt](mailto:dianasousa@deb.uminho.pt); [madalena.alves@deb.uminho.pt](mailto:madalena.alves@deb.uminho.pt))

\*\* Laboratory of Microbiology, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

**Abstract** This paper reviews recent results obtained on long-chain fatty acids (LCFA) anaerobic degradation. Two LCFA were used as model substrates: oleate, a mono-unsaturated LCFA, and palmitate, a saturated LCFA, both abundant in LCFA-rich wastewaters. 16S rRNA gene analysis of sludge samples submitted to continuous oleate- and palmitate-feeding followed by batch degradation of the accumulated LCFA demonstrated that bacterial communities were dominated by members of the *Clostridiaceae* and *Syntrophomonadaceae* families. Archaeal populations were mainly comprised of hydrogen-consuming microorganisms belonging to the genus *Methanobacterium*, and acetate-utilizers from the genera *Methanosaeta* and *Methanosarcina*. Enrichment cultures growing on oleate and palmitate, in the absence or presence of sulfate, gave more insight into the major players involved in the degradation of unsaturated and saturated LCFA. *Syntrophomonas*-related species were identified as predominant microorganisms in all the enrichment cultures. Microorganisms clustering within the family *Syntrophobacteraceae* were identified in the methanogenic and sulfate-reducing enrichments growing on palmitate. Distinct bacterial consortia were developed in oleate and palmitate enrichments, and observed differences might be related to the different degrees of saturation of these two LCFA. A new obligately syntrophic bacterium, *Syntrophomonas zehnderi*, was isolated from an oleate-degrading culture and its presence in oleate-degrading reactors was verified by fingerprinting analysis.

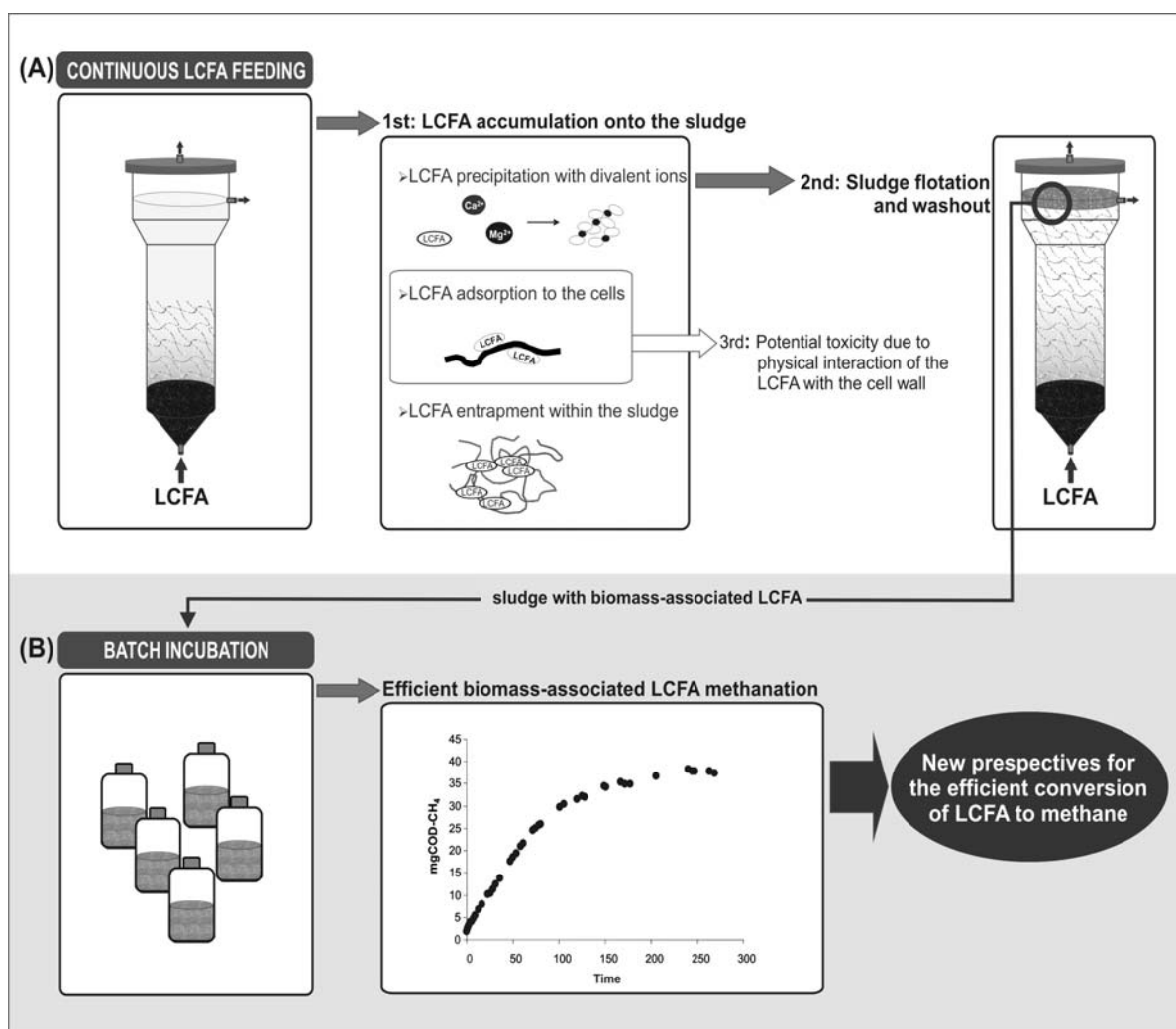
**Keywords** Anaerobic digestion; LCFA, oleate, palmitate, syntrophy

### Introduction

Wastewaters, particularly those from food processing industries, contain considerable amounts of long-chain fatty acids (LCFA). These compounds, resulting from the hydrolysis of oils and fats, are potentially attractive for biogas production because of their high potential methane yield. Yet, removal of LCFA from the wastewaters prior to anaerobic treatment is rather standard, which implies the loss of their energetic value. Reasons for this procedure are, basically, related with the recurrent reports on the alleged toxic/inhibitory effect of LCFA towards methanogenic activity (e.g. Hanaki *et al.*, 1981; Koster and Cramer, 1987; Rinzema *et al.*, 1994), as well as with problems of sludge flotation and washout during the treatment of LCFA-rich wastewater in high- rate anaerobic reactors (e.g. Rinzema, 1988; Hwu *et al.*, 1998a; Hwu *et al.*, 1998b) (Fig. 1A). However, studies conducted at our research group showed that the adverse effects of LCFA on anaerobic sludge functionality are not irreversible and that, under appropriate conditions, LCFA can be efficiently converted to methane (Pereira *et al.*, 2003; 2004) (Fig. 1B). Cycles of continuous feeding of lipid/LCFA-rich wastewaters followed by batch degradation of the accumulated substrate might be an appropriate way to treat this type of wastewater.

In methanogenic reactor systems, LCFA degradation proceeds via  $\beta$ -oxidation, yielding acetate and hydrogen, which are subsequently converted to methane and CO<sub>2</sub> (Weng and Jeris, 1976). The overall conversion involves the concerted action of LCFA-oxidizing bacteria and methanogenic archaea that utilize hydrogen and acetate (Schink, 1997). In environments where sulfate is present sulfate-reducing bacteria can oxidize LCFA to acetate and CO<sub>2</sub> (or ultimately only to CO<sub>2</sub>), with production of sulfide (Rabers *et al.*, 2000). Thus far, 10 acetogenic bacteria have been characterized that grow on fatty acids with more than 4 carbon atoms and up to 18 carbon atoms, in syntrophic association with methanogens. They all belong to the families *Syntrophomonadaceae* within the group of low G+C-containing Gram-

positive bacteria (McInerney, 1992; Zhao *et al.*, 1993; Wu *et al.*, 2006), or *Syntrophaceae* in the subclass of the  $\delta$ -Proteobacteria (Jackson *et al.*, 1999). Described sulfate-reducing LCFA-oxidizers are relatively more diverse and are distributed among 13 different genera within the *Desulfobacterales*, *Desulfuromonadales* and *Syntrophobacterales* orders (Rabers *et al.*, 2000).



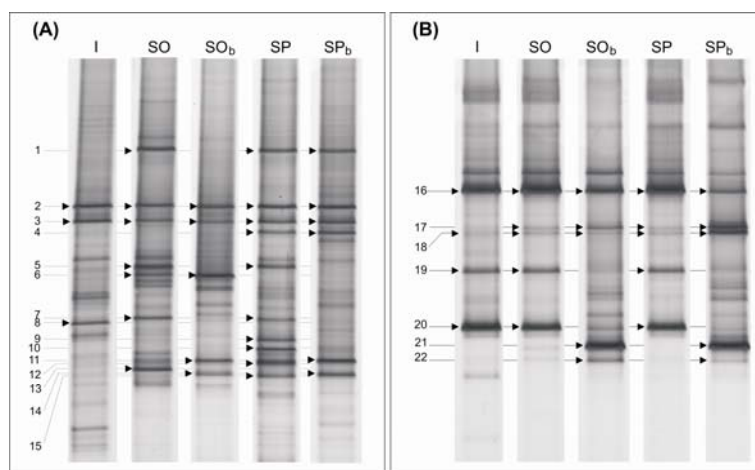
**Figure 1: Flow chart of the sequential LCFA degradation.** (A) When a lipid/LCFA-rich wastewater is fed to a continuous anaerobic reactor, a substantial accumulation of LCFA onto the sludge is observed. LCFA accumulation is progressive and, at long last, conversion to methane stops and the sludge, then enclosed by a whitish foam, starts to float and to washout from the reactor. These operational problems, associated with the theories of LCFA toxicity towards anaerobic communities, required lipids/LCFA removal from wastewaters before biological treatment. (B) Batch incubation of the LCFA-“loaded” sludge, without addition of other carbon or energy sources, demonstrated that this sludge is actually still able to convert the biomass-associated LCFA to high amounts of methane. These results contradict the findings about the severe and irreversible toxicity of LCFA and suggest that the apparent inhibition during continuous LCFA feeding is reversible. New perspectives for the efficient conversion of LCFA to methane, potentially based on a two-phase process (LCFA continuous accumulation followed by batch degradation), are prospected from these new insights.

Current knowledge on LCFA degradation is mainly derived from the study of pure cultures. A better understanding of the microbial diversity and function of LCFA-degrading communities in anaerobic reactors is still lacking. Insight about the phylogenetic affiliation of microorganisms involved in LCFA degradation can be investigated by molecular 16S rRNA gene-targeting techniques, which in combination with cultivation techniques might give a more detailed picture of the microbial communities directly involved in LCFA degradation. Linking microbial information with biotechnological advances might be crucial for the

development of new approaches enabling the efficient treatment of LCFA-rich wastewaters. Hence, this paper reviews some of the results obtained at our research group concerning microbiological aspects of the degradation of LCFA in anaerobic environments. Oleate and palmitate, the most abundant unsaturated and saturated LCFA present in wastewaters, respectively, were used as model substrates throughout the research.

#### LCFA-degrading microbial communities in methanogenic reactors

Microbial communities present in sludge samples submitted to a cycle of continuous LCFA-feeding followed by batch degradation of biomass-associated substrate were studied by using molecular techniques. A total of five sludge samples were compared: samples containing a high amount of accumulated LCFA, SO and SP, obtained after a continuous load in two EGSB reactors with oleate (unsaturated LCFA) and palmitate (saturated LCFA), respectively; the suspended sludge used as inoculum for both reactors, sample I; and samples SO<sub>b</sub> and SP<sub>b</sub>, obtained after batch incubation of sludge SO and SP, respectively, to allow the degradation of the accumulated LCFA. Predominant bacterial and archaeal phylotypes of the different samples were monitored using DGGE of PCR-amplified 16S rRNA gene fragments (Fig. 2). Composition of the predominant community visualized in the DGGE patterns was determined



by 16S rRNA gene sequencing of 22 clones (bands 1-22 Fig. 2, Table 1).

**Figure 2:** DGGE patterns of (A) bacterial and (B) archaeal amplicons obtained from the sludge samples: I - inoculum, SO – sludge after continuous load in EGSB reactor with oleate, SP- sludge after continuous load in EGSB reactor with palmitate, SO<sub>b</sub> – sludge SO after degradation of accumulated LCFA in batch, SP<sub>b</sub> – sludge SP after degradation of accumulated LCFA in batch.

Comparison of the DGGE band-patterns from sludge samples I, SO and SP revealed a significant shift in the composition of the bacterial community during the continuous load with LCFA (accumulation step). At the end of the continuous feeding, the bacterial community present in the reactors fed with oleate and palmitate exhibited a low resemblance (54% Pearson similarity between SO and SP). No significant changes in the archaeal communities were observed during LCFA accumulation, indicating that composition of the archaeal microbiota in the bioreactor remained rather stable (99% Pearson similarity between SO and SP). After batch degradation of the accumulated substrates changes in the bacterial profiles were observed with Pearson similarity indices for sludge samples SO/SO<sub>b</sub> and SP/SP<sub>b</sub> of 61 and 75%, respectively. Archaea profiles of both depleted sludges exhibited a lower similarity towards the respective LCFA-loaded sludge as compared to the bacterial microbiota (archaeal profile Pearson similarity indices for SO/SO<sub>b</sub> and SP/SP<sub>b</sub> were 37 and 6%, respectively).

**Table 1:** Affiliation of the retrieved bacterial (clones 1 to 15) and archaeal (clones 16 to 22) clones

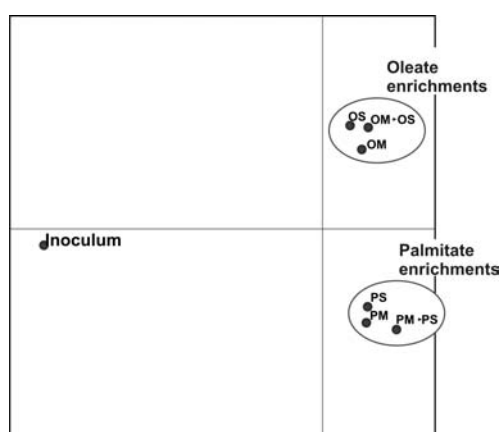
Band ID	Closest relative (>1200 bp)	% Identity	Phylum	Accession nos.
1	<i>Clostridium</i> sp 45	99	Firmicutes	DQ339700
2	<i>Clostridium butyricum</i>	99	Firmicutes	DQ339701
3	Uncultured bacterium clone C118	96	Firmicutes	DQ339702
4	Uncultured bacterium clone p-2117-s959-	99	Firmicutes	DQ339703
5	<i>Clostridium propionicum</i>	99	Firmicutes	DQ339704
6	Uncultured bacterium clone R6b2	94	Firmicutes	DQ339705
7	Uncultured bacterium H30	96	Proteobacteri	DQ339706
8	<i>Eubacterium callanderi</i>	89	Firmicutes	DQ339707
9	Uncultured bacterium Eub No 20	97	Firmicutes	DQ339708
10	Unidentified eubacterium clone	99	Firmicutes	DQ339709
11	<i>Syntrophomonas wolfei</i>	93	Firmicutes	DQ339710
12	Uncultured bacterium clone TSAT05	99	Firmicutes	DQ339711
13	Uncultured bacterium clone PL-7B6	98	Bacteroidetes	DQ339712
14	<i>C. butyricum</i> (NCIMB8082)	94	Firmicutes	DQ339713
15	<i>Syntrophomonas wolfei</i>	92	Firmicutes	DQ339714
16	<i>Methanobacterium formicicum</i>	98	Euryarchaeot	DQ339715
17	<i>Methanobacterium aarhusense</i>	97	Euryarchaeot	DQ339716
18	<i>Methanobacterium formicicum</i>	98	Euryarchaeot	DQ339717
19	<i>Methanosaeta concilii</i>	99	Euryarchaeot	DQ339718
20	<i>Methanosaeta concilii</i>	99	Euryarchaeot	DQ339719
21	<i>Methanosarcina mazei</i> strain Goe1	99	Euryarchaeot	DQ339720
22	<i>Methanosarcina mazei</i> strain Goe1	99	Euryarchaeot	DQ339721

In the overall LCFA-accumulation/degradation process a majority of the analyzed bacterial clones (87%) clustered within the *Firmicutes* phylum. A prevalence of microorganisms belonging to the *Clostridiaceae* (Fig. 2, bands 1, 2, 4, 5 and 14) and *Syntrophomonadaceae* (Fig. 2, bands 6, 10, 11 and 15) suggests that these populations play an important role in LCFA degradation. Also relevant is the fraction of the retrieved 16S rRNA gene sequences that are closely related to yet uncultured microorganisms (53% of the total sequences), suggesting that more work on the cultivation of LCFA-degrading bacteria should be done. Hydrogenotrophic archaea, liable on making the overall LCFA conversion thermodynamically favorable, were present in all the analyzed samples. Members of the *Methanobacterium* genus are predominant in both accumulation and degradation steps (Fig. 2, bands 16, 17 and 18). *Methanosaeta* spp were detected as predominant acetate-utilizers during continuous oleate and palmitate feeding (Fig. 2, bands 19 and 20) but, were virtually replaced by *Methanosarcina* spp after batch degradation of the accumulated substrate (Fig. 2, bands 21 and 22). This might be related with differences in the acetate concentration during the continuous feeding and batch degradation. In fact, acetate concentrations in the continuous reactors were rather low (below 300 mg L<sup>-1</sup>), favoring the dominance of *Methanosaeta* spp that have a high affinity for acetate (Jetten *et al.*, 1992). On the other hand, during batch degradation, the release of large amounts of acetate to the medium due to degradation of the biomass-associated LCFA might create favorable conditions for growth of *Methanosarcina* spp, which have a lower affinity for acetate but a higher growth rate than *Methanosaeta* spp (Jetten *et al.*, 1992).

#### Microbial communities present in oleate- and palmitate-enrichment cultures

LCFA-degrading communities were developed by selective enrichments growing on oleate (unsaturated LCFA) and palmitate (saturated LCFA), in the presence and absence of sulfate. The same inoculum sludge was used to start up four different enrichment series: OM and PM cultures, growing on oleate or palmitate, respectively, without the presence of a inorganic electron acceptor other than protons and CO<sub>2</sub> (favoring methanogenesis) and, OS and PS cultures, growing on oleate or palmitate, respectively, in the presence of sulfate (favoring sulfate reduction). Later on, stable methanogenic OM and PM were incubated in medium

containing oleate or palmitate, respectively, plus sulfate, and subjected to subsequent transfers with sulfate: OM-OS and PM-PS cultures. Changes in the microbial composition during enrichment were analyzed by DGGE profiling of PCR-amplified 16S rRNA gene fragments (data not shown). DGGE profiles of stable enrichment cultures were treated by principal component analysis (PCA) to evaluate distances between the different communities (Fig. 3). As result of the PCA analysis two marked clusters, relatively distant from the inoculum sludge, were obtained: one cluster containing the different oleate-enrichment cultures and the other with the palmitate-enrichment cultures. These results suggest that the substrate used during enrichment had a major influence in the bacterial community structure and a stronger shifting effect than the presence or absence of sulfate in the environment. One reason that might partially explain the differential clustering of the bacterial populations present in oleate- and palmitate-enrichment cultures is the fact that the two LCFA used have different degree of chain saturation. Oleate is an unsaturated LCFA with a double bond at position C9, while palmitate has a completely saturated chain. In fact, only a minority of the characterized LCFA-degrading bacteria is able to degrade unsaturated LCFA, indicating that this is a specific feature of some microorganisms (Sousa, 2007).



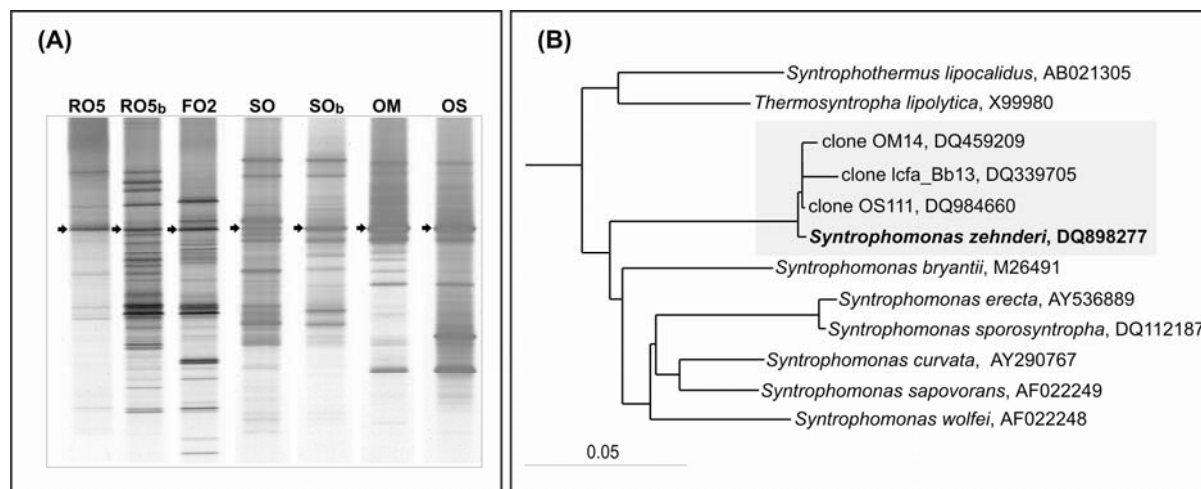
**Figure 3:** PCA of DGGE profiles obtained after specific 16S rRNA gene amplification of genomic DNA from stable oleate- and palmitate-enrichment cultures: OM – methanogenic oleate-enrichment (12 transfers); PM – methanogenic palmitate-enrichment (12 transfers); OS – sulfate-reducing oleate-enrichment (7 transfers); PS – sulfate-reducing palmitate-enrichment (7 transfers); OM → OS – methanogenic oleate-enrichment after 4 successive transfers in medium containing sulfate; PM → PS – methanogenic palmitate-enrichment culture after 4 successive transfers in medium containing sulfate. PC1 and PC2 represent 40.3% and 30.5% of the variation, respectively.

Prominent DGGE-bands of the enrichment cultures were identified by 16S rRNA gene sequencing. A significant part of the retrieved 16S rRNA gene sequences was most similar to those of uncultured bacteria. 16S rRNA gene sequences clustering within the *Syntrophomonadaceae* family were identified as corresponding to predominant DGGE-bands in the oleate- and palmitate-enrichment cultures (OM, OS, PM and PS). In both stable palmitate-enrichment cultures (PM and PS) members of the *Syntrophobacteraceae* family were present. In the enrichment cultures grown in the presence of sulfate (OS and PS) sulfate-reducing bacteria closely affiliated with *Desulfovibrio*, *Desulfomicrobium* and *Desulforhabdus* genera were also detected. These bacteria are probably involved in the use of the hydrogen and acetate resulting from LCFA oxidation, in a similar role taken by methanogenic archaea when no sulfate is present in the medium.

#### ***Syntrophomonas zehnderi*, a novel syntrophic LCFA-degrading bacterium linked to oleate degradation**

A novel LCFA-degrading bacterium, *Syntrophomonas zehnderi*, was isolated as a co-culture with *Methanobacterium formicicum* from an anaerobic bioreactor treating an oleate-based effluent (Sousa *et al.*, 2007). This mesophilic, syntrophic, fatty acid oxidizing bacterium degrades straight-chain fatty acids with 4 to 18 carbon atoms but, also, unsaturated LCFA, such as oleate (in co-culture with *M. formicicum*). Microorganisms closely related to *Syntrophomonas zehnderi* in sludges degrading oleate under different conditions, i.e.

continuous load, fed-batch operation and enrichment series were detected by DGGE analysis (Fig. 4). The presence of *Syntrophomonas zehnderi* related bacteria in sludges after contact with oleate suggests its important role in anaerobic oleate degradation in bioreactor sudge. Moreover, the fact that these microorganisms are present in the oleate-degrading sludges but not in palmitate-degrading sludges suggests their direct link to oleate degradation.



**Figure 4:** (A) DGGE patterns of bacterial amplicons from the sludge obtained after: continuous load in bio-reactor with oleate (at a loading rate up to 8 Kg COD m<sup>-3</sup> day<sup>-1</sup>) – RO5; batch degradation of the substrate accumulated in RO5 – RO5<sub>b</sub>; successive feed-batch loads with oleate – FO2; continuous load in bioreactor with oleate (at a loading rate up to 4 Kg COD m<sup>-3</sup> day<sup>-1</sup>) – SO; batch degradation of the substrate accumulated in SO – SO<sub>b</sub>; methanogenic enrichment series with oleate – OM; and, enrichment series with oleate in the presence of sulfate – OS. (B) Phylogenetic tree showing the position of the 16S rRNA gene clones retrieved from the different sludge samples in contact with oleate (DQ339705, DQ459209 and DQ98466) within representatives of fatty acid degrading bacteria.

## Conclusions

Application of cultivation and molecular techniques to the study of microbial composition of LCFA-degrading sludges provided important insight into the communities involved in the degradation of these compounds. Members of the *Clostridiaceae* and *Syntrophomonadaceae* appeared pivotal to LCFA-degradation. Oleate- and palmitate-degrading cultures showed a different microbial composition, indicating that the types of bacteria in the community might depend on the saturation degree of the fed LCFA. A novel LCFA-degrading bacterium, *Syntrophomonas zehnderi*, was isolated from an oleate-enrichment culture and could be linked to oleate degradation under several conditions. A proposal for genome sequencing of this bacterium is accepted by the DOE-JGI and future genomic comparison of this strain with *S. wolfei*, which only degrades saturated LCFA, will provide more insight into the differences in the degradation of unsaturated and saturated LCFA.

## Acknowledgments

The authors acknowledge the Fundação para a Ciência e Tecnologia (FCT) and Fundo Social Europeu (FSE) for the financial support given to D. Z. Sousa (SFRH/BD/8726/2002) and M. A. Pereira (SFRH/BPD/14591/2003).

## References

Hanaki K., Nagase M., and Matsuo T. (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnology and Bioengineering* **23** (7), 1591-1610.

- Hwu C.-S., van Lier J. B., and Lettinga G. (1998a) Physicochemical and biological performance of expanded granular sludge bed reactors treating long-chain fatty acids. *Process Biochemistry* **33** (1), 75-81.
- Hwu C.-S., Tseng S. K., Yuan C. Y., Kulik Z., and Lettinga G. (1998b) Biosorption of long-chain fatty acids in UASB treatment process. *Water Research* **32** (5), 1571-1579.
- Jackson B. E., Bhupathiraju V. K., Tanner R. S., Woese C. R., and McNerney M. J. (1999) *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Archives of Microbiology* **171** (2), 107-114.
- Jetten M. S. M., Stams A. J. M., and Zehnder A. J. B. (1992) Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiology Reviews* **88** (3-4), 181-197.
- Koster I. W. and Cramer A. (1987) Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. *Applied and Environmental Microbiology* **53** (2), 403-409.
- McNerney M. J. (1992) The genus *Syntrophomonas*, and other syntrophic bacteria. In: Balows A., Trüper H. G., Dworkin M., Harder W., and Schleifer K. H. (eds), *The Prokaryotes*. 2048-2057, Springer, New York.
- Pereira M. A., Cavaleiro A. J., Mota M., and Alves M. M. (2003) Accumulation of long chain fatty acids onto anaerobic sludge under steady state and shock loading conditions: effect on acetogenic and methanogenic activity. *Water Science and Technology* **48** (6), 33-40.
- Pereira M. A., Sousa D. Z., Mota M., and Alves M. M. (2004) Mineralization of LCFA associated with anaerobic sludge: kinetics, enhancement of methanogenic activity, and effect of VFA. *Biotechnology and Bioengineering* **88** (4), 502-511.
- Rabers R., Hansen T., and Widdel F. (2000) Dissimilatory sulfate- and sulfur-reducing prokaryotes. In: Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., and Stackebrandt E. (eds), *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, Springer-Verlag, New York.
- Rinzema A. (1988) Anaerobic treatment of wastewater with high concentrations of lipids or sulfate. Wageningen Agricultural University, The Netherlands.
- Rinzema A., Boone M., van Knippenberg K., and Lettinga G. (1994) Bactericidal effect of long chain fatty acids in anaerobic digestion. *Water Environmental Research* **66** 40-49.
- Schink B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* **61** (2), 262-280.
- Sousa D.Z. (2007) Ecology and physiology of microbial communities that degrade long-chain fatty acids. University of Minho, Portugal.
- Sousa D. Z., Smidt H., Alves M. M., and Stams A. J. M. (2007) *Syntrophomonas zehnderi* sp. nov., an anaerobe that degrades long chain fatty acids in co-culture with *Methanobacterium formicicum*. *International Journal of Systematic and Evolutionary Microbiology* **57** 609-615.
- Weng C. and Jeris J. S. (1976) Biochemical mechanisms in methane fermentation of glutamic and oleic acids. *Water Research* **10** (1), 9-18.
- Wu C., Liu X., and Dong X. (2006) *Syntrophomonas erecta* subsp. *sporosyntrophica* subsp. nov., a spore-forming bacterium that degrades short chain fatty acids in co-culture with methanogens. *Systematic and Applied Microbiology* **29** (6), 457-462.
- Zhao H., Yang D., Woese C. R., and Bryant M. P. (1993) Assignment of fatty acid-b-oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16S rRNA sequence analysis. *International Journal of Systematic Bacteriology* **43** 278-286.